4-day cultured cells appears to be T3-associated, it is likely that, within the fresh Lyt2$^-$, L3T4$^-$ population, this receptor is predominantly expressed on the T3$^+$ subset of Lyt2$^-$, L3T4$^-$ thymocytes. Are these T3$^+$ cells the precursors to mature T cells bearing αβ and, if so, does the γ chain have a direct role in the thymic influence on receptor repertoire development? To postulate such a role, one would need to invoke a two-receptor model (*21, 22*) in which the receptor containing the γ-chain was positively selected in the thymus and then reexpressed on mature T cells as a second receptor (in addition to αβ) with self-MHC specificity. The N-glycosidase experiments are at variance with such a model; the fact that all of the thymically expressed γ chain is N-glycosylated indicates a total absence of the $V_{1.2}$-C_2 combination, which is the major γ mRNA expressed in mature clones and peripheral T cells. Furthermore, neither the anti-T3, nor the anti-γ precipitated any γ protein from the surface of three activated mature cytotoxic lymphocyte (CTL) clones that transcribe full-length γ mRNA (*20*).

Because $V_{\gamma 2}$-$C_{\gamma 1}$ rearrangements are seen in mature T cells, our data do not completely rule out the precursor nature of T3$^+$, Lyt2$^-$, L3T4$^-$ thymocytes. It is possible that all of the thymic 35-kD γ chain is derived from the $V_{\gamma 2}$-$C_{\gamma 1}$ rearrangement, which is subsequently not transcribed in mature T cells, or that $V_{\gamma 3}$-$C_{\gamma 1}$ and $V_{\gamma 4}$-$C_{\gamma 1}$ rearrangements are expressed in thymocyte precursors and then deleted by a V-gene replacement with $V_{\gamma 2}$ (*4, 23*). However, we favor the more likely possibility that these cells represent a distinct thymus-dependent lineage. This subpopulation may regulate the differentiation of T3$^-$, Lyt2$^-$, L3T4$^-$ precursors to mature αβ receptor-bearing cells. Alternatively, they may be exported and exert their effector function extrathymically. It is now important to determine the functional characteristics of this TCR γ-bearing thymocyte subpopulation.

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Determination of Junction Avidity of Cytolytic T Cell and Target Cell

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A direct measurement of the avidity of the junction between a cytotoxic T lymphocyte and its target cell was achieved by using a biophysical approach. A micromanipulation technique was used to determine the force required to separate a cytotoxic T cell (human clone F1, with specificity for HLA-DRw6) from its specific target cell (JY: HLA-A2, -B7, -DR4, w6) prior to delivery of the lethal hit. The force required to separate the F1-JY pair is 1.5 × 10^4 dynes per square centimeter. This junction avidity for F1-JY pairs is 6 to 13 times greater than that for F1-F1 and JY-JY pairs; the F1-JY conjugate requires a stronger separating force and is more easily rejoined than the homologous cell pairs. This study provides an estimate of the avidity of cytotoxic T cells for their target cells and insights into the biophysical correlates of the molecular complexes formed in the interaction of cytotoxic T cells and their targets during the cytotoxic process.

THE MOLECULAR MECHANISM OF cell-mediated cytolysis has been the subject of several investigations (*1–5*). Long-term allospecific human cytolytic T lymphocyte (CTL) lines have been developed and characterized and are useful for the study of the mechanism of killing at the molecular and cellular level (*6–9*). The interdigitation of the plasma membranes at the contact region between the CTL and its target cell (TC) (*10, 11*), and the changes in the centrioles, the Golgi system (*12*), and the microfilamental-microtubular networks (*13, 14*) have been examined by electron microscopy and immunofluorescence microscopy.

Specific antigen and receptor interaction between a CTL and its TC leads to a reorientation of actin microfilaments and microtubules; this interaction can be inhibited by monoclonal antibodies (*15–18*). The first step of cell-mediated cytolysis is the conjugation of a CTL with its specific TC, and this CTL-TC conjugation could be a commitment step for cytolysis. Our aim was to use the micromanipulation technique with micropipettes to perform quantitative measurements under direct microscopic observation on single CTL-TC pairs, in order to deduce the interaction forces between

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CTL surface receptors and TC surface antigens. This approach can provide information on the junctional avidity between single CTL-TC pairs rather than on the percentage of conjugation of CTL's in an overall population (*19*).

The target cell we used, JY (HLA-A2,2; B7,7; DR4,w6), is an Epstein-Barr virus–transformed B lymphoblastoid cell line. JY is maintained in RPMI 1640 medium (M.A. Bioproducts, Bethesda, Maryland) supplemented with 10% heat-inactivated fetal calf serum (M.A. Bioproducts), 2 m*M* L-glutamine (Gibco, Grand Island, New York), penicillin at 100 unit/ml, and streptomycin at 100 μg/ml (Gibco), 10 m*M* Hepes buffer (M.A. Bioproducts), and 25 μ*M* 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, New York).

F1 cytotoxic T lymphocytes were prepared from mononuclear cells obtained from the peripheral blood of a normal donor (HLA-A11,Aw32; B27,Bw51; Cw2, DR7, 7; Genox 3.53$^-$). The mononuclear cells were separated on a Ficoll-Hypaque gradient (lymphocyte separation medium; Bionetics, Kensington, Maryland) at 600*g*, washed repeatedly and stimulated with irradiated JY cells (10,000 R) as described by Krensky *et al.* (*7*). Bulk CTL cultures were maintained in the supplemented RPMI 1640 medium for 3 to 4 weeks with weekly JY stimulation and then cloned by limiting dilution at less than one cell per well in flat-bottom 96-well microtiter plates (Linbro, Flow Laboratories, McLean, Virginia) with 1×10^5 to 2×10^5 irradiated JY cells per well and human conditioned medium containing 10% interleukin 2 (IL-2). Clones were expanded in 16-mm wells, characterized for cytolytic function and specificity, and subcloned.

Assays for cytotoxicity were performed in triplicate in V-bottom 96-well microtiter plates. JY cells were labeled with Cr-51 ($Na_2{}^{51}CrO_4$, New England Nuclear, Boston, Massachusetts) and plated at 1×10^3 cells per well with varying numbers of F1 effector cells in a total volume of 150 μl of RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, Hepes buffer, and 2-mercaptoethanol. The assay plates were centrifuged at 200*g* for 5 minutes and incubated at 37°C. After 4 hours of incubation, plates were again centrifuged and 100-μl portions of cell-free supernatants were counted in a gamma counter to determine the amount of ^{51}Cr released. Cytolytic activity is calculated as the percentage of specific release (SR) of ^{51}Cr: $SR = 100 \times [E - C)/(T - C)]$, where E is the radioactivity (in counts per minute) released from target cells by incubation with effector cells, T is the total radioactivity released from target cells with 5% Triton X-100 detergent, and C is the spontaneous radioactivity released from target cells by incubation with medium alone.

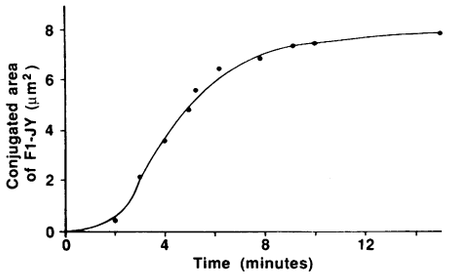

Fig. 1. Change of the area of conjugation with time for one F1-JY cell pair.

Micropipettes with internal radii (R_p) of 2.8 to 3.4 μm, for holding the JY cell, and 1.8 to 2.0 μm, for holding the F1 cell, were prepared with the use of a micropipette puller and filled with the culture medium. Each micropipette was mounted on a hydraulic micromanipulator, with the wide end of the pipette connected to a pressure regulation system. The detailed arrangement has been described elsewhere (*20–23*). The F1 and JY cells were each held at the tip of a micropipette by using a small aspiration pressure (P; approximately 100 to 500 dyn/cm^2). The F1 and JY cells were brought close together and aligned by manipulating the holding pipettes. The negative pressure in the F1-holding pipette was removed, and the F1 cell was allowed to interact freely with the JY cell still held by its holding pipette. About 10 minutes after the beginning of conjugation, the F1-holding pipette was manipulated to recapture the F1 cell

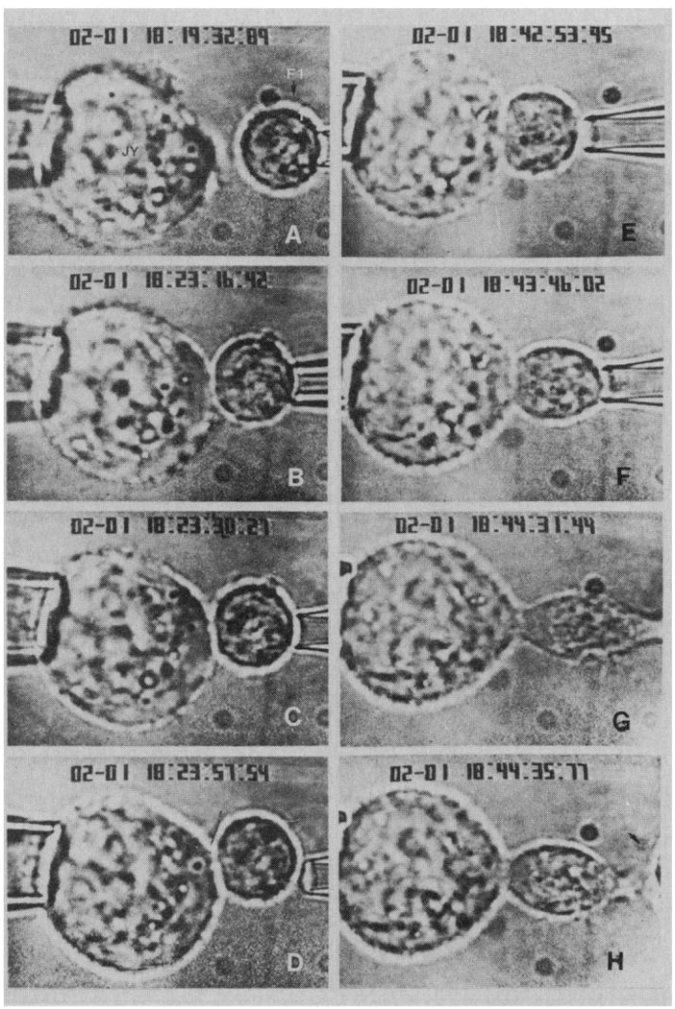

Fig. 2. Sequence of photographs taken from the TV monitor. The numbers on the top of the screen represent, in order, month, day, hours, minutes, seconds, and tens of milliseconds. (A) The F1 and JY cells were held by two different-sized pipettes with negative aspiration pressure. The pipette diameter and aspiration pressure were smaller for F1 than for JY. (B and C) The pipette holding the F1 cell was moved by micromanipulation to allow F1 to conjugate with the JY cell. (D) Within 1 minute, F1 and JY were joined; the conjugation continued after release of the negative pressure on F1 and withdrawal of the pipette. (E) After a stable cell conjugation was attained, F1 was again held by the pipette and the two pipettes were aligned by micromanipulation. (F and G) F1 was pulled away from JY by moving the smaller pipette with the micromanipulator. Deformation of both cells increased as they were partially separated. (H) The applied pressure was not sufficient to hold the F1 cell for complete separation of two cells. F1 slid away from the smaller pipette, and another cell (arrow showing its edge) flowed toward the pipette in response to the negative pressure.

with an aspiration pressure lower than that in the JY pipette.

Within 10 to 15 minutes after the beginning of conjugation, the aspiration pressure in the F1-holding pipette was increased stepwise at increments on the order of 100 dyn/cm^2. At each pressure level, the F1-holding pipette was pulled away gradually (at a rate of approximately 0.5 μm/sec) by micromanipulation. For low pressures, such pulling caused the F1 cell to slip out of its holding pipette while remaining conjugated with JY. With sufficiently high pressure, the F1-JY conjugate became completely separated as the F1-holding pipette was being pulled away from JY; each cell remained attached to its holding pipette. The minimum aspiration pressure that led to the total separation of the two cells is referred to as the critical separation pressure (P_c). The critical separation stress (S_c) was calculated as

$$S_c = 2(R_p/R_i)^2 P_c \quad (1)$$

where R_p is the radius of the F1-holding pipette and R_i is the radius of the interface of conjugation at rest; $2(R_p/R_i)^2$ gives the ratio of the surface area of the portion of the cell aspirated (a hemispherical cap with area = $2\pi R_p^2$) to the conjugation area (πR_i^2). Throughout the experiment, the force resultant (aspiration pressure × pipette radius) was always larger for the JY-holding pipette than for the F1-holding pipette. The pressure regulatory device used had an accuracy of better than 5 dyn/cm^2 and a time constant of approximately 20 msec (*20*). These studies were performed at room temperature and *p*H was kept at 7.4 with Hepes buffer. A total of 17 measurements were made on nine F1-JY cell pairs. Similar experiments were also performed on 33 F1-F1 cell pairs and 35 JY-JY cell pairs.

We determined that the CTL clone F1 was specific for HLA-DRw6 by using a panel of known HLA-specific target cells; those targets expressing HLA-DRw6 were lysed by F1. At an F1 to JY ratio of 10:1, the specific release of ^{51}Cr was consistently about 60% and the spontaneous release was about 15%.

In the resting state, the diameter of the F1 cell was approximately 8 μm and that of the JY cell was 13 to 19 μm. After the F1 and JY pair had been aligned with micropipettes, the F1 cell was allowed to interact with the JY cell held by its holding pipette. The length of the conjugation region between the two cells as seen on the video screen increased with time until a steady level of 2.5 to 3.0 μm was reached in 8 to 15 minutes. If the conjugated region is assumed to be a disk, with its diameter considered to be the length of conjugation, the area of

Table 1. Experimental data on F1-JY separation after conjugation.

Experiment	Radius of holding pipette (μm)		Radius of conjugation area (μm)	Critical separation	
	JY	F1		P_c (10^4 dyn/cm^2)	S_c (10^4 dyn/cm^2)
1	3.37	1.65	1.46	0.60	1.53
			1.46	0.65	1.66
2	2.60	2.40	1.46	0.29	1.57
			1.47	0.30	1.60
3	3.70	1.65	1.33	0.50	1.54
			1.37	0.53	1.54
4	2.37	1.38	1.32	0.88	1.92
5	3.65	1.65	1.47	0.60	1.51
			1.46	0.55	1.41
6	3.65	1.70	1.59	0.54	1.23
			1.53	0.50	1.24
7	3.65	1.20	1.42	0.90	1.29
			1.43	0.96	1.35
8	3.10	1.30	1.39	1.00	1.75
			1.38	0.98	1.74
9	2.40	1.45	1.36	0.70	1.59
			1.44	0.75	1.52

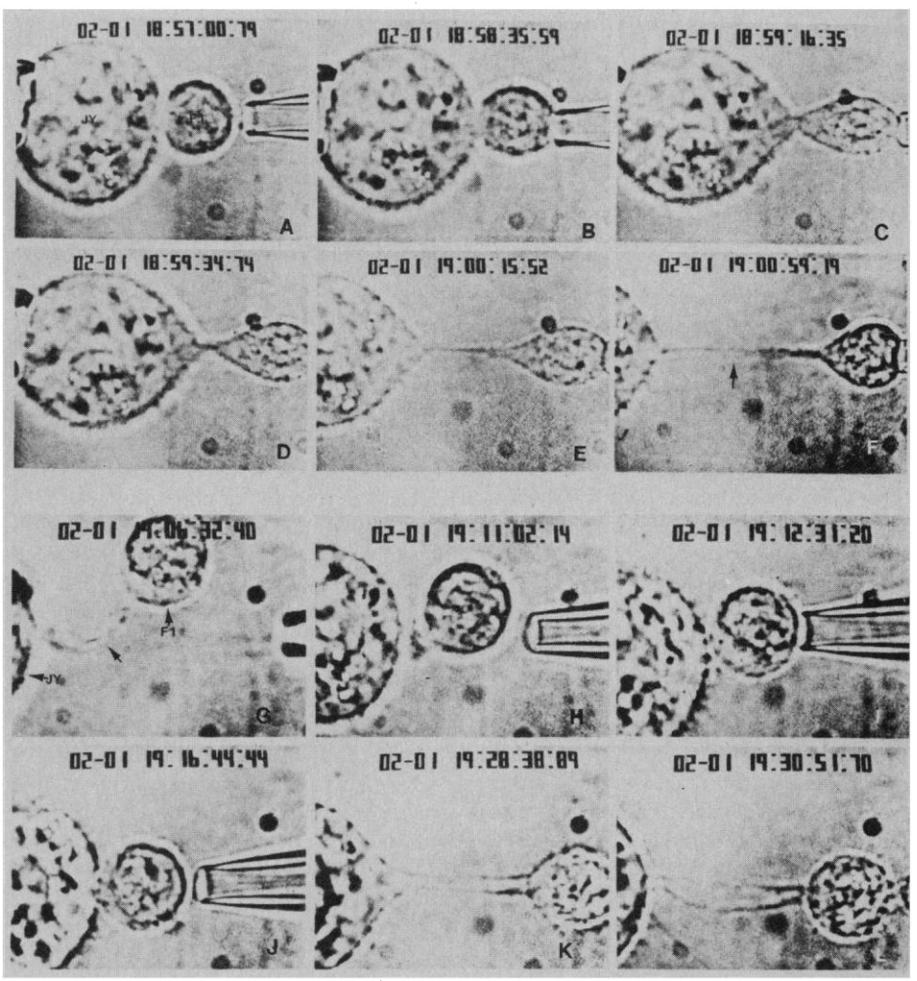

Fig. 3. An experiment similar to that in Fig. 2, but with a higher applied pressure used to hold the F1 cell. (A) Conjugation of JY and F1 cells when the F1 cell was released from its holding pipette. (B) Reaspiration of F1 and alignment of the two pipettes. (C and D) F1 was pulled away from JY, and both cells showed deformation. (E and F) The F1 cell was increasingly separated from the JY cell as the F1-holding pipette was pulled away. The conjugated area was reduced to membrane tethers, probably as a result of membrane interdigitation between these two cells. (G) The pressure in the F1-holding pipette was released, and F1 was free in medium, only with some membrane tether (arrow) attached to JY. (H and I) F1 was gradually drawn close to JY as the membrane tether disappeared. (J) F1 reconjugated with JY. (K and L) F1 was again pulled away from JY; the force requirement was comparable to that of the first trial; that is, the experiments are reproducible.

conjugation at steady state averaged 6.5 μm^2 (calculated from the radius of conjugation area as listed in Table 1). The time course of the development of the conjugation area between one F1-JY pair is shown in Fig. 1.

The force required to separate the F1-JY pair was tested by stepwise increases of the negative pressure in the F1-holding pipette and pulling of this pipette away from the conjugated area (Fig. 2). At low aspiration pressures there was no separation of the two cells, and they remained conjugated during the pulling process; the F1 cell slipped out of its holding pipette when the distance of pulling was too far (>5 μm). When pulling was performed at higher pressures, the two cells were deformed (elongated) (Fig. 2), and a tether began to form between them (Fig. 3). Whether the conjugated cells separate or not depended primarily on the pressure level rather than the rate of pulling. For $P < P_c$, the pulling away of the F1-holding pipette resulted in the slipping of the F1 cell out of the pipette and the resumption of the original conjugation shape, with disappearance of the tether (Fig. 3). At $P \geq P_c$, pulling of the F1-holding pipette caused the complete separation of the two cells.

The results of 17 measurements on nine F1-JY pairs are shown in Table 1. Repeated measurements on the same cell pairs, which had been separated and allowed to reconjugate in approximately the same location, yielded results with good agreement. The critical separation stress for F1-JY conjugates was found to be 1.5×10^4 dyn/cm^2. This is six times stronger than the critical separation stress for F1-F1 and 13 times that for JY-JY conjugates. After their complete separation by mechanical force, the F1-JY pairs were more easily rejoined (Fig. 3) than JY-JY or F1-F1 pairs.

In some experiments, the conjugation of F1 with JY was allowed to continue till lysis. Microscopic observations on these single cell pairs indicate that the F1-JY conjugation persisted throughout the entire cytotoxic process, even after the lysis of the JY cell.

Conjugation of CTL's with their targets is the first step of the cell-mediated killing process. With the two-pipette technique, the force of interaction between CTL's and TC's has been estimated from the pressure required to separate a pair of conjugated F1-JY cells. The elongation of F1 and JY cells and the tether formation with rising pressure levels below P_c indicate that the force required for separating these cells is greater than that required for their deformation. The force required to separate conjugated F1-JY pairs is approximately six times that needed to separate F1-F1 and 13 times that for JY-JY. These results suggest that the F1-JY pair has specific conjugation forces that are much stronger than the self F1-F1 or JY-JY pairs. The good agreement in results obtained on repeated measurements of F1-JY pairs (Table 1) indicates that both cells are viable after separation. This is important in view of the fact that the separation of some other types of cell conjugates could involve membrane rupture and cell damage (*24*).

Table 2. Critical separation stress for two-cell conjugation. Values are means ± SEM for N experiments. Analysis of variance showed that the results on F1-JY were significantly different from those for F1-F1 and for JY-JY.

Cell pair	N	Critical separation stress (10^3 dyn/cm^2)
F1-JY	17	15.29 ± 0.45
F1-F1	33	2.58 ± 0.02
JY-JY	35	1.18 ± 0.03

The time period involved in cell separation (<2 minutes) is much shorter than that in conjugation (8 to 15 minutes). These results indicate that conjugation and disjunction are not symmetric processes (*25*). A longer time is required for the interaction between the receptors and antigens than for their disjunction, probably because of the lateral movements of these proteins in the cell membranes to strategic positions. The shorter time required for the rejoining of F1-JY pairs immediately after their separation may be a result of the receptors and antigens having already been brought to favorable positions to allow their ready interaction. Our studies on single F1-JY pairs are in agreement with other reports (*26, 27*) indicating that conjugation persists even after JY lysis.

Our results yield a critical separation stress of 15.29×10^3 dyn/cm^2 for F1-JY conjugates. Bell (*28*) has derived a critical force of 4×10^{-6} dyn per bond for cell conjugates formed by antigen-antibody interaction. Combination of these results suggests that there are 40 bonds per square micrometer. This number is smaller than the density of ~500 bonds per square micrometer found by Berke (*4*) for CTL-TC conjugates. There are several possible explanations for this discrepancy. First, the critical force derived by Bell (*28*) may not necessarily be directly applicable to the CTL-TC system studied. Second, the cell lines used in the present investigation are not the same as those studied by Berke (*4*). Finally, the area occupied by the conjugating bonds is difficult to determine. On the one hand, the complicated membrane interdigitation (*4, 26*) may increase the interfacial area between the two cells over that measured under the light microscopy; on the other hand, only a small fraction of the interfacial area is actually occupied by the bonding molecules (*28*).

Our investigation has established the biophysical basis of the specific interactions of F1-JY cell pairs. We have provided a new approach for investigating the structural and functional determinants of cytotoxic interactions at the single-cell level. Experiments of this kind can be applied to assess quantitatively the roles of different types of molecules and their functional groups in various stages of cytotoxic killing, including recognition, conjugation, programming, and lethal hit delivery. For example, investigations on such interactions with the use of monoclonal antibodies to various surface antigens and receptors would help to elucidate the molecular basis of the interaction between CTL's and TC's and the cytolytic processes.

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